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EFFECTS OF OXYGEN AND GLUTATHIONE ON THE OXYGEN CONSUMPTION AND SUCCINATE DEHYDROGENASE ACTIVITY OF LIVER

By

Dennis A. Baeyens, Ph.D.
Mary J. Meier, B.S.

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US ARMY AEROMEDICAL RESEARCH LABORATORY
Fort Rucker, Alabama 36362

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The effects of hyperbaric oxygen tensions on the oxygen consumption and succinate dehydrogenase (SDH) activity of mouse liver were investigated. Mouse liver homogenate exposed to a P_{O_2} of 3837.8 mm Hg for 30 minutes showed a 50.6% reduction in oxygen consumption compared to controls exposed to nitrogen at ambient pressure. The SDH activity of mouse liver was significantly reduced after a 3 hour exposure to a P_{O_2} of 3796 mm Hg.

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The effect of glutathione as a protective agent against oxygen toxicity was also examined. Liver pretreated with reduced glutathione and exposed to high oxygen tensions demonstrated greater activity than untreated controls. Oxidized glutathione protected SDH against hyperbaric oxygen toxicity. It is concluded that glutathione can stimulate oxygen consumption and maintain SDH activity after exposure to hyperbaric oxygen by increasing succinate formation through the glutathione-succinate shunt.

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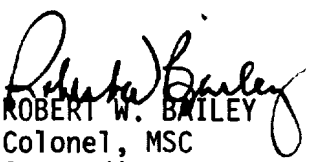
SUMMARY

The US Army Aeromedical Research Laboratory is evaluating the effects of oxygen on the US Army aircrew. The basic cellular response of enzyme production and function to oxygen has not been fully defined. Oxygen at decreased and increased pressure has produced significant brain and hepatic enzyme alterations. This study evaluated the effect of increased oxygen tensions on oxygen consumption and succinate dehydrogenase (SDH) activity of mouse hepatic tissue.

Hepatic oxygen consumption decreased 50.6% after thirty minutes at P_{O_2} of 3837.8 mm Hg. SDH was reduced after 3 hours exposure. Pretreatment with reduced or oxidized glutathione prevented the SDH reduction. This study supports the concept of protection from oxygen toxicity by increased production of succinate through the glutathione-succinate shunt.

Through understanding and clear definition of cellular enzyme function under alternobaric conditions, aeromedical research can be fully applied to operational oxygen problems.



ROBERT W. BAILEY
Colonel, MSC
Commanding

EFFECTS OF OXYGEN AND GLUTATHIONE ON THE OXYGEN CONSUMPTION AND SUCCINATE DEHYDROGENASE ACTIVITY OF LIVER

INTRODUCTION

In 1878, Bert (4) initially observed that oxygen at high pressures inhibited both the catabolism of sugar and oxygen consumption in mammalian tissues. In more recent studies, high pressure oxygen was shown to rapidly inhibit α-ketoglutarate dehydrogenase and pyruvic oxidase activity in rat brain mitochondria (16). Kaplan and Stein (8) demonstrated depressed metabolic functions in brain tissue slices exposed to hyperbaric oxygen. The hypothesis that oxygen exerts its toxic action on tissues by inhibiting adenosine triphospate (ATP) production and thus the energy available for maintaining cell functions was proposed. These observations led to the conclusion that efforts to prevent cellular oxygen toxicity should be directed toward maintaining ATP concentration.

Studies of oxygen toxicity *in vitro* have provided support for the hypothesis that oxidation of sulfhydryl groups of important tissue enzymes plays a role in the production of the symptoms of oxygen toxicity *in vivo* (7). Oxidation of sulfhydryl groups could either occur within a molecule or between different molecules resulting in disulfide bridge formation and consequent enzyme inactivation. One group of sulfhydryl containing enzymes, the dehydrogenases, has been shown to be particularly sensitive to the toxic effects of oxygen. An important glycolytic enzyme, lactate dehydrogenase (LDH), from the amphibian retina was shown to be inhibited by exposure to oxygen at normabaric pressures for 24 hours (2). Succinate dehydrogenase, an enzyme involved in the oxidative tricarboxylic acid (TCA) cycle, was shown to be inhibited by agents that react with sulfhydryl groups, and this enzyme has also been shown to be inactivated by oxygen *in vitro* (15). Inhibition of the enzymes involved in oxidative metabolism would be reflected by a decreased cellular oxygen consumption upon exposure to high oxygen tensions. In most *in vitro* studies dealing with oxygen toxicity, the inhibiting effects of oxygen on cellular metabolism have been demonstrated only after exposure of tissue slices or homogenates to oxygen for either long periods of time or to extremely high oxygen tensions.

In the cell, reduced glutathione is a major sulfhydryl group containing component. Reduced glutathione (GSH) is known to activate a number of enzymes. It was proposed by Barron (3) that the main function of this substance is to maintain sulfhydryl groups of enzymes in their reduced and active state. The early rationale for using GSH as a protective agent against oxygen toxicity was mainly this "sulfhydryl sparing effect" (3). More recently, it has been observed that glutathione may stimulate oxidative metabolism by another mechanism (12). Glutathione can be converted to succinate in a number of different tissues. This increased production of succinate can serve as a secondary support system for the maintenance of high energy levels.

This study of the effects of oxygen at alternobaric pressure on hepatic oxygen consumption and succinate dehydrogenase content was undertaken to define their basic cellular metabolic mechanisms. Through this knowledge physiologic and therapeutic modalities can be utilized in support of operational research for the protection of US Army aircrews.

MATERIALS AND METHODS

Oxygen Consumption Study--Female Swiss-Webster mice (6 months old, approximate weight 35-40g) were used in all experiments. Mice were killed by cervical dislocation and approximately 850 mg of liver was removed and weighed to the nearest 0.1 mg. The hepatic tissue was homogenized in 5.5 ml of distilled water for 15 seconds. A 2-ml sample of the homogenate was placed in each of two 50-ml Erlenmeyer flasks containing 22 mg of dextrose and 20 ml of mammalian Krebs-Ringer solution (pH 7.60, osmolarity 289.25 mOsm/kg modified to contain 2.94 g $NaHCO_3$). In addition, 78 mg of reduced glutathione (Calbiochem, Los Angeles, CA) was added to one of the flasks making the final concentration of GSH equal to 12 m M/l. Two 10-ml portions were then withdrawn from each flask and placed in four separate petri dishes. The two control petri dishes, one containing GSH and the other without GSH, were exposed to a nitrogen environment [PN_2=757 mm Hg (N=16)] for 30 minutes. The two experimental petri dishes, one containing GSH and the other without GSH, were placed in a table-top Bethlehem Environmental Chamber (Model H-70-A Bethlehem, PA). The hyperbaric chamber was filled with medical oxygen at a pressure of 3837.8 mm Hg. The total incubation time was 30 minutes, including a 15 minute purge with oxygen at a flow rate of 8 l/minute to completely remove residual gases.

The oxygen consumption of the control and experimental tissue homogenates was determined by means of a YSI Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, OH).

The temperature of the system was regulated at 20 ± 0.1°C by a constant temperature water bath. The electrode was calibrated against an air-saturated solution of modified mammalian Krebs-Ringer of 3 ml volume contained in one chamber of the tissue bath assembly. The other three chambers each contained 3 ml of the air-saturated liver homogenate. To determine the oxygen consumption, the meter readings were taken at the start and finish of a 5 minute recording period. By subtracting the concentration of oxygen remaining after 5 minutes from the initial concentration of oxygen, and knowing the solubility coefficient of oxygen in mammalian Krebs-Ringer (α=0.0310) (18) at 20°C, it was possible to determine the oxygen consumption of the tissue homogenate. Oxygen utilization was expressed as µl of oxygen consumed per hour per mg wet weight. All values were corrected to standard temperature and pressure, dry (STPD). Student t-test was used for statistical analysis.

Succinate Dehydrogenase Study--A portion of mouse liver weighing approximately 450 mg was rapidly excised and blotted dry on filter paper. The sample was divided into two approximately equal portions and each portion was accurately weighed to the nearest 0.1 mg. To one portion, 0.1 ml of phosphate buffer (0.034 M, pH 7.4) was added for each 5 mg of tissue. To the other portion, 0.1 ml of phosphate buffer containing 12 m M/l oxidized or reduced glutathione was added for each 5 mg of tissue. The pH of the phosphate buffer containing glutathione was adjusted to 7.4. Both samples were then homogenized for 15 seconds and centrifuged (Beckman, model LZ-50 ultracentrifuge) at 4°C, 20,000 rpm for 5 minutes.

For incubation, the supernatant was divided into 100 µl samples and each sample was placed in 1 ml capacity glass incubation vials. Each vial was tightly capped to prevent evaporation. Each cap was pierced by an 18 gauge hypodermic needle for the purpose of pressure equalization during the hyperbaric exposure.

The incubation vials were divided into control and experimental groups. Each group contained eight vials with glutathione and eight vials without glutathione. The control group was exposed to ambient barometric pressure [P_{O_2}=158.1 (13) mm Hg] at 20°C for 3 hours. The experimental group was exposed to 3794.9 (13) mm Hg P_{O_2} for 3 hours at 20°C.

The SDH activities were determined by measuring the spectral reduction of ferricyanide at a wavelength of 400 mµ at 20°C (9). The assay medium consisted of 0.3 ml of potassium cyanide (0.1 M, pH 7.0), 0.3 ml potassium ferricyanide (0.01 M), 0.4 ml sodium succinate (0.5 M), and 2.0 ml of phosphate buffer (0.034 M, pH 7.4). At zero time 25 µl of liver homogenate was added to the assay medium in a quartz cuvette and thoroughly mixed. The changing optical density was compared to a blank containing distilled water for 2 minutes.

Enzymatic activity is expressed as µ moles of succinate converted per minute per mg protein. Protein determinations were accomplished by a modification of the method of Lowry (10). Multiple comparisons of the means of enzyme activities utilized the Newman-Keuls statistical test. Values considered significant for these studies have a p value of 0.05 or less.

RESULTS

Oxygen Consumption Studies--Preliminary experiments were performed to determine if exposing the control liver homogenates to 100% nitrogen at ambient pressures had a detrimental effect on oxygen consumption. There were no significant differences ($P>0.1$) in oxygen consumption between homogenates exposed to room air and those exposed to nitrogen

at ambient pressure for 30 minutes. The reducing nitrogen environment was chosen as a precaution against any possible metabolic oxidations that might occur in the control homogenates.

Table I provides the mean values for liver oxygen consumption from 16 animals. Statistical analysis revealed no significant differences ($P>0.1$) in liver oxygen consumption between animals. The toxic effect of oxygen on oxidative metabolism was demonstrated by a 50.6% reduction in oxygen consumption of the experimental homogenates exposed to hyperbaric oxygen for 30 minutes. This reduction was significant at a $P>0.01$.

Control homogenates pretreated with GSH showed significantly higher ($P>0.01$) oxygen consumption than control homogenates containing no GSH. Experimental homogenates pretreated with GSH and exposed to hyperbaric oxygen also had higher rates of oxygen consumption ($P>0.01$) than untreated controls. Thus, GSH not only protected liver tissue against hyperbaric oxygen toxicity but also stimulated oxygen consumption over that of the untreated controls.

Succinate Dehydrogenase Studies--Succinate dehydrogenase activity was significantly inhibited after exposure to hyperbaric oxygen. Control preparations of the study of effects of reduced glutathione (Table II) and oxidized glutathione (Table III) demonstrate a 13.5% reduction and 10.7% reduction of SDH activity.

The enzyme preparations incubated with either oxidized or reduced glutathione maintained their activity after exposure to hyperbaric oxygen. Glutathione did not significantly increase the activity of SDH in the control homogenates indicating it has no direct stimulatory effect on the enzyme.

DISCUSSION

There is abundant evidence that respiration of tissue slices and homogenates is depressed by oxygen at pressures above 1 ATM (7). This inhibitory effect on overall cellular metabolism has been demonstrated to occur in many tissues, with brain being the most sensitive (5). However, as pointed out by Haugaard (7), there has been a reluctance to conclude that the inhibitory effects of oxygen on metabolism are the direct cause of the symptoms of oxygen toxicity in the intact animal. The reason for this reluctance is that inactivation of enzymes *in vitro* appeared to be too slow in onset to account for the symptoms of oxygen toxicity in the intact animal. Our study indicates that these earlier views may have been too conservative. Oxygen is capable of reducing the oxygen consumption of liver by 50.6% in as little time as one-half hour at a relatively low pressure.

TABLE I: Oxygen consumption of mouse liver as influenced by hyperbaric oxygen. Oxygen utilization determinations were made after 30 minutes at 20°C and were expressed in terms of µl of oxygen consumed per hour per mg wet weight.

TISSUE PREPARATION	INCUBATION GAS	O_2 CONSUMPTION Mean ± S.E.(N)	% CHANGE***
CONTROL LIVER HOMOGENATE	P_{N_2} = 756.7 mm Hg	63.25 ± 2.86(48)*	---
CONTROL LIVER HOMOGENATE WITH GSH (12mM)	P_{N_2} = 756.7 mm Hg	109.17 ± 2.61(48)	+ 42.1
EXPERIMENTAL LIVER HOMOGENATE	P_{O_2} = 3837.8 mm Hg	31.27 ± 1.94(48)**	- 50.6
EXPERIMENTAL LIVER HOMOGENATE WITH GSH (12mM)	P_{O_2} = 3837.8 mm Hg	98.50 ± 3.36(48)	+ 35.8

GSH = Reduced glutathione.
*Significantly different from GSH treatments at 1% level.
**Significantly different from untreated control at 1% level.
***Based on % change from untreated control.

TABLE II: Hepatic succinate dehydrogenase activity as influenced by hyperbaric oxygen and reduced glutathione. Succinate dehydrogenase activity determinations were made after 3 hours at 20°C and were expressed as μ moles/min/mg protein.

Specimen and Incubation Media	P_{O_2} mm Hg	SDH Activity Mean ± S.E.(N)	% Change*
Liver Homogenate with Phosphate Buffer	755.6	1.031 ± 0.01838(24)	--
Liver Homogenate with Phosphate Buffer and GSH (12mM)	755.6	0.9558 ± 0.01612(24)	- 7.3
Liver Homogenate with Phosphate Buffer	3795.6	0.8925 ± 0.01330(24)	-13.5**
Liver Homogenate with Phosphate Buffer	3795.6	1.002 ± 0.01215(24)	- 2.9

SDH = Succinate dehydrogenase.
GSH = Reduced glutathione.
*Based on % change from room air control without reduced glutathione.
**Significant at the 5% level.

TABLE III: Hepatic succinate dehydrogenase activity as influenced by hyperbaric oxygen and oxidized glutathione. Succinate dehydrogenase activity determinations were made after 3 hours at 20°C and were expressed as μ moles/min/mg protein.

Specimen and Incubation Media	P_{O_2} mm Hg	SDH Activity Mean ± S.E.(N)	% Change*
Liver Homogenate with Phosphate Buffer	756.7	0.9421 ± 0.01048(24)	--
Liver Homogenate with Phosphate Buffer and ox glu (12mM)	756.7	0.9779 ± 0.01939(24)	+ 3.7
Liver Homogenate with Phosphate Buffer	3796.7	0.8417 ± 0.01021(24)	-10.7**
Liver Homogenate with Phosphate Buffer and ox glu (12mM)	3796.7	0.9725 ± 0.01465(24)	+ 3.2

Ox glu = oxidized glutathione.
*Based on % change from room air control without oxidized glutathione.
**Significant at the 5% level.

Numerous studies have demonstrated that various sulfhydryl group containing compounds can afford protection against oxygen toxicity. Baeyens (1) has shown that GSH can maintain LDH activity even after exposure of mouse brain homogenates to a P_{O_2} of 5764 mm Hg for 5 hours. The observation that GSH, as well as other substances containing sulfhydryl groups, has a protective effect against oxygen toxicity provides support for the concept that oxidation of sulfhydryl groups plays a role in the mechanism of oxygen toxicity. Reduced glutathione protects important tissue constituents by a "sulfhydryl sparing effect"; that is, by maintaining sulfhydryl groups in a reduced and viable state.

In a study dealing with glutathione as a possible protective agent against oxygen toxicity, the glutathione-GABA-succinate pathway has been implicated as a support system for the maintenance of brain ATP levels in six species of experimental animals exposed to hyperbaric oxygen (13). This pathway, also called the glutathione-succinate shunt, is shown in Figure 1. In another report, this same sequence of reactions has been demonstrated to occur in mouse liver (11). The physiological significance of the shunt has not been clearly elucidated, but two possible functions have been proposed (13). First, it has been suggested that the shunt serves as a means of metabolizing γ-aminobutyric acid (GABA). A second possibility is that the shunt functions in bypassing the inhibition of α-ketoglutarate from the cycle by transamination with GABA to yield glutamate and the re-entry of the carbon chain at the succinate level. The possible physiological significance of the glutathione-succinate shunt may be as an alternate source for the production of succinate.

It has been shown that oxidized glutathione protected mice against oxygen toxicity *in vivo* but to a lesser degree than did GSH (6). In our studies oxidized glutathione maintained the activity of SDH after exposure to an oxygen tension that would otherwise be toxic to this enzyme. Since the sulfhydryl sparing effect would not be a contributing factor with oxidized glutathione, other means of protection must occur. It is proposed that the glutathione-succinate shunt is providing the added means of protection demonstrated in this study. The glutamic molecule could be split off the oxidized glutathione and provide protection through the increased production of succinate. High levels of the substrate succinate may be necessary to maintain the activity of SDH throughout the incubation period.

Experiments in which rats were exposed to high pressure oxygen have shown that prior injections of succinate resulted in normal and above normal concentrations of ATP being present in the cerebral hemispheres, liver and kidney (15). In contrast, in the corresponding tissues of animals not pretreated with succinate, there was a significant reduction in the concentration of ATP. It was concluded that succinate protects against oxygen toxicity by restoring ATP concentration and metabolic function in these tissues.

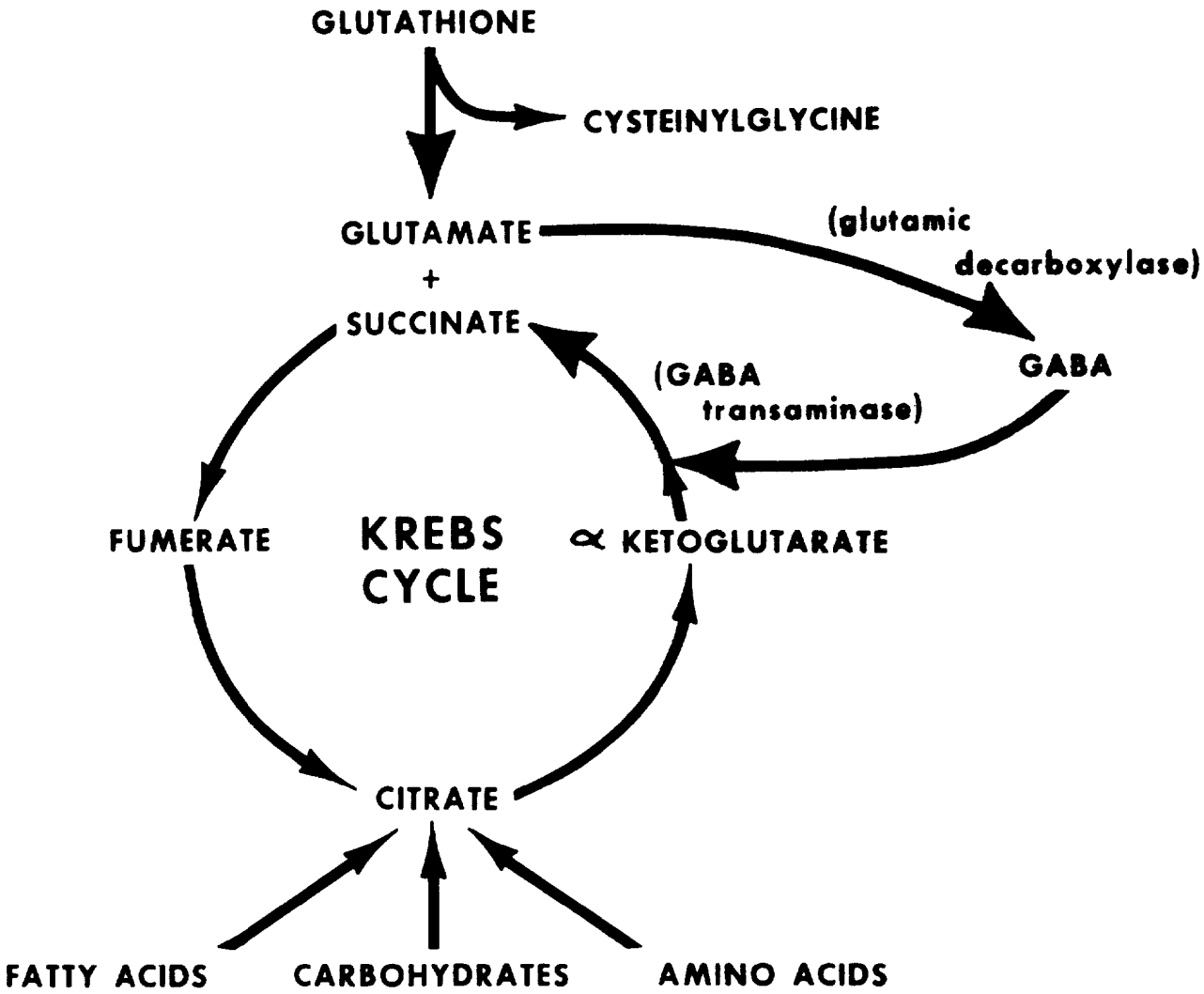


FIGURE 1.

GSH-Succinate Shunt (shown by large arrows) and its relationship to the Krebs cycle.

Succinate has been found to have a higher ATP production capacity in rat brain, liver and kidney than did either glutamate and α-ketoglutarate (14). With succinate as substrate, only two molecules of ATP are formed per molecule of oxygen used as compared to three molecules of ATP per molecule of oxygen when α-ketoglutarate is converted to succinate by the Krebs cycle. Thus, succinate not only causes high production of ATP but also utilizes more oxygen than other oxidative phosphorylation substrates.

This finding is the mechanism producing the increased oxygen consumption of the liver homogenates treated with GSH in our experiments. GSH increases succinate production by means of the glutathione-succinate shunt resulting in increased oxygen consumption by the tissue to maintain the energetic efficiency of ATP formation.

Both GABA and glutamic decarboxylase, the enzyme which catalyzes the formation of GABA from glutamic acid, were found to be present in the gray matter of the central nervous system (12) and in hepatic tissue (11). Wood and Watson (18) found a significant reduction in brain levels of GABA after exposing rats to 100% oxygen at a pressure of 6 ATM absolute for 33 minutes. They hypothesized that oxygen inhibits the Krebs cycle at the α-ketoglutaric dehydrogenase step and that the GABA succinate shunt becomes the major route of metabolism necessitating a greater requirement for GABA. If the enzyme systems responsible for the formation of GABA cannot cope with the increased demand, the concentration of GABA in the brain will decrease, and the oxidation by the GABA-succinate shunt would be inadequate. It was thus concluded that the toxicity of high pressure oxygen in the brain was due to both an inhibition of α-ketoglutaric dehydrogenase and to a shortage of GABA and succinate as substrates. In our experiments there was not a shortage of GABA or succinate due to their increased formation from glutathione by way of the glutathione-succinate shunt, and consequently there was no impairment of oxidative metabolism in mouse liver homogenates even after treatment with hyperbaric oxygen.

In conclusion, the 50.6% reduction in hepatic oxygen consumption indicates that hyperbaric oxygen can inactivate an enzyme or enzymes of oxidative metabolism. Glutathione has no direct stimulatory effect on SDH but protects the enzyme against oxygen inactivation by maintaining a critical level of succinate formation during periods of exposure to hyperbaric oxygen. The fact that liver homogenates pretreated with GSH demonstrated higher rates of oxygen consumption than the homogenates without GSH was due to increased succinate production by means of the GSH-succinate shunt.

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